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High mortality in *Bufo gargarizans* eggs associated with an undescribed *Saprolegnia ferax* strain in the Republic of Korea

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ABSTRACT: Pathogenic water molds have a significant impact on many species, especially amphibians. The genus *Saprolegnia* is a pathogenic oomycete restricted to aquatic and moist habitats, and its presence is strongly linked to the abundance of amphibians and fishes. We investigated the influence of *Saprolegnia* presence on egg mortality and egg occurrence under varying environmental conditions in the Asiatic toad *Bufo gargarizans* at 27 breeding sites in the Republic of Korea. We then assessed the impact of *Saprolegnia* on the presence of *B. gargarizans* at the 27 sites surveyed weekly during the *B. gargarizans* breeding season for 3 consecutive years. We used molecular tools to identify the water molds as belonging to an undescribed *S. ferax* strain. We demonstrated that the presence of *S. ferax* was positively associated with higher water conductivity and ponds. In addition, while *S. ferax* prevalence was associated with a reduction in *B. gargarizans* breeding activity and breeding success, we could not determine its impact on the subsequent breeding seasons. Our study highlights the potential negative effects of *Saprolegnia* on amphibian reproduction, although additional research is necessary to determine the relationship between *Saprolegnia*, its hosts and the impacts of habitat loss on amphibians.

KEY WORDS: Water mold · Oomycete · *Saprolegnia ferax* · Amphibians · Water quality · Toad conservation

1. INTRODUCTION

Anthropogenic activities have led to substantial changes in climatic conditions, sometimes resulting in increased pathogen prevalence (Daszak et al. 2001, Anderson et al. 2004). For instance, increases in mean temperatures (Hoegh-Guldberg et al. 2018), pH (Caldeira & Wickett 2003), UV-B radiation (Beebee & Griffiths 2005), extreme drought periods (Kohli et al. 2019) and cloud cover (Pounds & Puschendorf 2004, but see Rohr et al. 2008) have shifted ecological balances and resulted in an increase in prevalence of pathogens and parasites on amphibians (Daszak et al. 2003, Pounds et al. 2006, Bancroft et al. 2008).

Members of the genus *Saprolegnia* (hereafter collectively referred to as *Saprolegnia*), an oomycete, are one of the pathogens implicated in massive amphibian mortality events (Banks & Beebee 1988, Beattie et al. 1991, Blaustein et al. 1994, Kiesecker & Blaustein 1995). *Saprolegnia* is known to kill larvae of *Rana aurora* (Romansic et al. 2006) and newly metamorphosed *R. cascadae* (Romansic et al. 2007) and has helped drive local extirpations of *R. pipiens* and *Anaxyrus terrestris* in North America (Bragg & Bragg 1958, Bragg 1962). Furthermore, *Saprolegnia* infection is associated with higher mortality rates and reduced hatching time in multiple amphibian species (Bragg & Bragg 1958, Bragg 1962, Walls & Jaeger

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1987, Gomez-Mestre et al. 2006, Perotti et al. 2013, Ghirardi et al. 2018). *Saprolegnia* is also a harmful pathogen for numerous fish species globally, particularly in hatchery settings (e.g. Seymour 1970, Pickering & Willoughby 1982, Bangyeekhun et al. 2001, van West 2006). In addition to succumbing to the pathogen, fishes possibly also serve as *Saprolegnia* vectors and sometimes spread the disease to amphibians (Kiesecker et al. 2001b).

The lethal effects of *Saprolegnia* on amphibian embryos can be amplified by numerous environmental factors, including pH, UV-B radiation, temperature and lower water depth due to lower precipitation (e.g. Beattie et al. 1991, Kiesecker & Blaustein 1995, Kiesecker et al. 2001a). For example, UV-B radiation may weaken the defense system of amphibians, resulting in increased vulnerability to pathogens such as *Saprolegnia* (Blaustein et al. 1994, Kiesecker & Blaustein 1995). Amphibian vulnerability to pathogens may also increase with water temperature, given the positive relationship between water temperature and the growth of *Saprolegnia* on anuran eggs (e.g. Gomez-Mestre et al. 2006).

The Asiatic toad *Bufo gargarizans* is a common bufonid species in Northeast Asia (Borzée et al. 2017b), but populations in the Republic of Korea (hereafter Korea) are declining due to urbanization-related habitat loss (IUCN SSC Amphibian Specialist Group 2019). Several sympatric anuran species are declining for similar reasons and are also susceptible to pathogens (Borzée et al. 2017a, 2018, Kwon et al. 2017). Unfortunately, the impacts of environmental conditions on these virulent amphibian pathogens (e.g. *Saprolegnia*) are so far poorly known. This results in a limit in the effective management of pathogen-mediated amphibian declines, despite the urgency for conservation activities in Korea (Borzée et al. 2019b). The aims of this study were to (1) identify the impact of *Saprolegnia* on the breeding activity of *B. gargarizans,* (2) determine the influence of *Saprolegnia* on subsequent breeding activities at the same site, (3) investigate the relationship between *Saprolegnia* and ecological variables, and (4) identify *Saprolegnia* species using molecular tools.

2. MATERIALS AND METHODS

2.1. Focal species

Saprolegnia (family Saprolegniaceae) is both saprobic and parasitic and is a pathogenic oomycete found in most aquatic habitats and moist soils. *Saprolegnia* molds infect a wide variety of organisms, including turtles, fish, insects and amphibians (e.g. MacGregor 1921, Seymour 1970, Bangyeekhun et al. 2001, van West 2006, Wolinska et al. 2008). This pathogen obtains nutrients from rotting organic matter and living hosts, which results in host infections (Seymour 1970, Noga 1993, Hussein & Hatai 2002). *Saprolegnia* can spread via contact from growing hyphae to immobile hosts such as amphibian egg masses or through colonization by free-swimming zoospores (Wood & Willoughby 1986). In Korea, 2 *Saprolegnia* strains have been reported to date: *S. diclina* on adult *Pelophylax chosenicus* and *Rana huanrenensis*, and *S. australis* on *P. chosenicus* tadpoles (Kim et al. 2008).

2.2. Data collection

We surveyed *Bufo gargarizans* weekly at 27 sites for at least 3 and up to 7 consecutive wk for each breeding season (27 February–25 April) between 2016 and 2018. As *B. gargarizans* is generally an explosive breeder and because of thermal differences due to altitudinal and latitudinal variations, hatching time was different between sites, and the number of surveys at a site reflected the developmental period and the latency in breeding. We started weekly surveys a week prior to the breeding season of the year before so as not to miss the beginning of the breeding season. The study sites (Fig. 1) comprised 5 ponds and 22 lakes distributed across Korea. To distinguish between ponds and lakes, we followed the limnologic definition where vegetation could grow over the totality of the pond's surface, while lakes are too deep to be totally covered by vegetation (Biggs et al. 2005).

For each survey at a study site, we recorded *B. gargarizans* presence or absence using visual and call-based evidence of breeding activity and the presence and number of egg strings. We recorded the number of egg clutches following the number of aggregations present, with an egg clutch considered independent when not connected by any egg string to another egg clutch. We adopted this protocol because female toads move during oviposition and because the number of egg string aggregations is an adequate proxy for the intensity of the breeding activity. Furthermore, we visually assessed the prevalence of *Saprolegnia* infections on each egg string aggregation encountered. Infections are visible in the form of a white cotton-like appearance on or in proximity of eggs, and *Saprolegnia* sp. identification was clarified

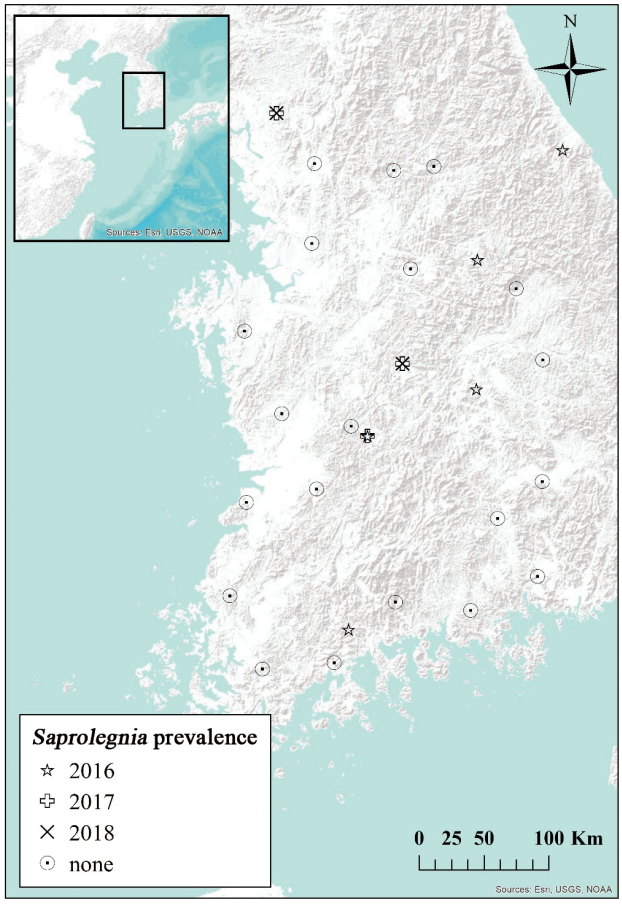

Fig. 1. Sampling locations to investigate the impact of *Saprolegnia* on the breeding activity of *Bufo gargarizans* in Korea between 2016 and 2018

under the microscope every time an egg clutch had *Saprolegnia* sp. symptoms (Fernández-Benéitez et al. 2008; Fig. 2). While *Saprolegnia* was likely present at all sites, we considered a site infected by *Saprolegnia* only when infection was above the threshold where it shows the physical symptoms of water mold infection. In 2016, the presence or absence (binary encoded) of *Saprolegnia* infection symptoms was recorded at each site, while in 2017 and 2018 we recorded both presence or absence and the percentage of clutches with symptoms. We then estimated the hatching success based on the percentage of eggs that had not hatched by the end of the usual hatching period. Non-hatched eggs become white and start decomposing, while the egg jelly opacifies, which makes the assessment of hatching success determinable without invasive examination.

We terminated the surveys when all embryos had hatched or were found dead. For each survey at each site, we recorded water conductivity (µS; measure of total amount of ions and total dissolved solids), water temperature (°C), water pH, dissolved oxygen (DO; ppm; PCSTestr 35 multimeter; Oakton Instruments), air temperature (°C), relative humidity (%; HT-350 thermo-hygrometer; Iondo), air pressure (HPa; DA-302 VGEBY altimeter; Xiami), photoperiod (indicated by sunset time; hh:mm; Sunrise Sunset app; alokm.com) and moon illumination (%; Phases of the Moon app; M2Catalyst). We also estimated percent cloud cover (10% increment) upon arrival at each study site and recorded habitat type (lake or pond, limnologic definition) at sites where breeding was apparent. Upon arrival at each water body, we searched for *B. gargarizans* egg clutches, tadpoles and adults by slowly walking at the water's edge. Surveys in 2016 determined the side of the water body where the species was breeding. For 2017 and 2018, small ponds were surveyed for the totality of their perimeter, while only the area where the species was breeding was surveyed for lakes, approximately 100 m in length.

2.3. DNA extraction, PCR amplification and sequencing

To ensure correct species identification in 2017, a representative egg clutch with infection symptoms was sampled once and preserved in 70% ethanol until use. Before DNA extraction, the sample was rinsed twice with 100% ethanol to remove organic debris, and mycelia were carefully detached from the egg surface using a sterilized scalpel and tweezers. Genomic DNA of the mycelia was extracted using a modified cetyl trimethylammonium bromide method (Rogers & Bendich 1994). The nuclear ribosomal internal transcribed spacer (ITS) region was amplified with the primer set ITS1oo (Riit et al. 2016) and ITS4 (White et al. 1990). PCR amplification was conducted using an AccuPower HotStart PCR PreMix kit (Bioneer) with 1 µl of template DNA and 1 µl of each primer. PCR denaturation was conducted at 95°C for 5 min, and amplification was done over 35 cycles at 95°C for 30 s, 55°C for 30 s and 72 °C for 40 s, followed by a final extension at 72°C for 10 min. The PCR products were checked on 1% agarose gel via electrophoresis and purified using an ExpinTM PCR SV kit (GeneAll Biotechnology). All sequencing was conducted at Cosmogenetech (Seoul, Korea).

The sequence was proofread and edited using MEGA5 (Tamura et al. 2011). Initial identification was performed using BLAST against the NCBI data-

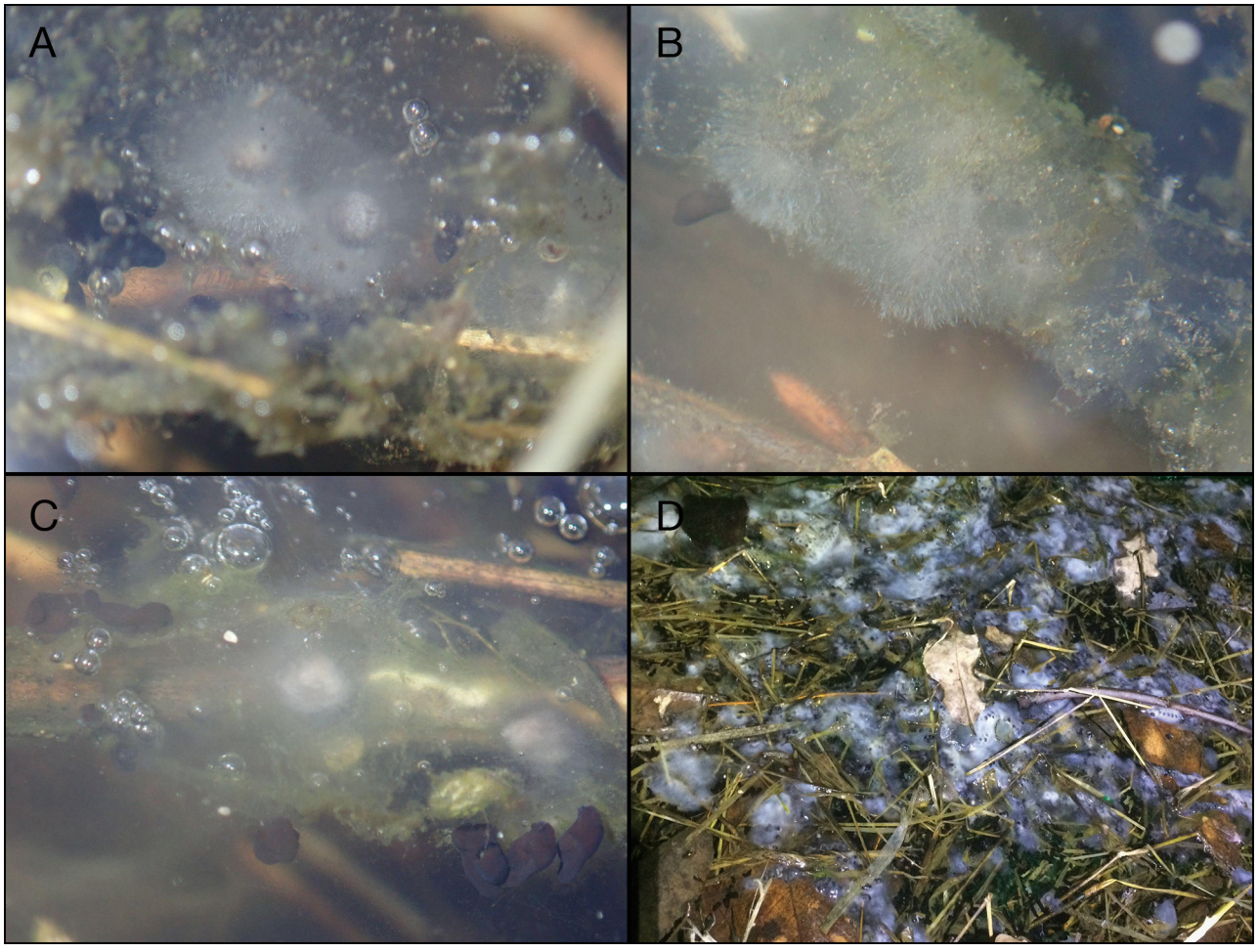

Fig. 2. *Bufo gargarizans* eggs infected with *Saprolegnia*, which has a white cotton-like appearance on and around the eggs. (A–C) Recently hatched tadpoles in proximity of eggs; (D) broader view of *Saprolegnia* infection in multiple egg clutches

base, and *Saprolegnia* was chosen as the candidate genus following the molecular operational taxonomic unit (mOTU) naming system suggested by Sandoval-Sierra et al. (2014). The sequence was then aligned with reference sequences of *Saprolegnia* mOTUs (Sandoval-Sierra et al. 2014) using MAFFT v.7 (Katoh & Standley 2013). The phylogenetic analysis was conducted using a maximum likelihood approximation in RAxML v.8 (Stamatakis 2014) with a GTR-GAMMA model and 1000 bootstrap replicates. We deposited the sequence of *Saprolegnia* in GenBank (accession number MK372991).

2.4. Statistical analysis

All statistical analyses were conducted in SPSS v.21.0. We used numerical encoding for sites (1 to 27) and habitat (lake or pond) and binary encoding for the occurrence of adults, eggs, tadpoles and *Saprolegnia*. Of the 417 points collected (see Supplement at www.int-res.com/articles/suppl/d137p089_supp.xlsx), some covariates were missing for 29 datapoints. We excluded egg occurrence from our analyses, as it was significantly correlated with the number of egg string aggregations (Pearson correlation; $n = 412$, $r = 0.30$, $p < 0.001$). Time was removed from the analyses because of the significant correlation with sites (time of day, Pearson correlation; $n = 407$, $r = 0.12$, $p = 0.018$). Similarly, air temperature, air pressure and ice cover were removed from analyses, as they were significantly correlated with water temperature (Pearson correlation; respectively, $n = 393$, $r = 0.62$, $p < 0.001$; $n = 402$, $r = 0.12$, $p = 0.013$; and $n = 403$, $r = 0.20$, $p < 0.001$). Because DO was correlated with moon luminosity and cloud cover (Pearson correlations; respectively, $n = 264$, $r = 0.26$, $p < 0.001$ and $n = 403$, $r = 0.23$, $p < 0.001$), it was also removed from the analyses.

First, we ran an analysis to test if *Saprolegnia* prevalence (i.e. percentage of infected clutches) had an impact on the presence of any breeding adults, eggs or tadpoles. We used a non-parametric related samples Cochran's *Q* test with adult presence, egg presence and tadpole presence as dependent variables, weighted by *Saprolegnia* prevalence in the form of infected clutches at a site over the breeding season.

We then tested whether the breeding output (i.e. presence of eggs) in a specific year was influenced by the occurrence (i.e. presence or absence) of *Saprolegnia* at the site the year prior. For this analysis, we used *Saprolegnia* presence at a site over the whole season (binary encoded) as the predictor variable. The analysis was conducted through repeated measures ANOVA with 3 levels (2016, 2017 and 2018), using *Saprolegnia* presence as a covariate in the between-subjects main effect model. The test met all necessary assumptions (Mauchly's test of sphericity; $W = 0.88$, df = 2, p = 0.253), and no outliers were detected.

Third, we used a binary logistic regression to determine which variables were related to *Saprolegnia* presence (n = 417 datapoints) after confirming that model assumptions were met (Box & Tidwell 1962). The binary logistic regression was set with *Saprolegnia* presence at a site for each survey independently as the dependent variable (n = 417), and we used the year, air humidity, water temperature, water conductivity, water pH, moon illumination, photoperiod, survey date and cloud cover as covariates in the model. Site and habitat were entered as categorical covariates in the model. To avoid autocorrelation of the model, as the surveys at a site were not independent from the surveys conducted at the same site at other dates, we nested the date of the survey within a site. We used a forward stepwise-entered model without constant so that all variables were included in the first block of the equation.

3. RESULTS

3.1. Relationship between *Saprolegnia* presence and toad breeding success

The highest number of sites with *Saprolegnia* presence was in 2016 (Table 1). We sampled 27 sites between 3 and 7 times each, resulting in a total of 139 visits (n_{survey}), and we recorded 5 sites as *Saprolegnia* positive ($n_{positive\ sites}$) over 8 surveys during which *Saprolegnia* symptoms were observed on egg clutches ($n_{Saprolegnia}$). The second highest number of sites was found in 2017 (n_{survey} = 130, $n_{positive\ sites}$ = 3, $n_{Saprolegnia}$ = 7), followed by 2018 (n_{survey} = 148, $n_{positive\ sites}$ = 2, $n_{Saprolegnia}$ = 2; Table 1). In 2016, there was an average of 1.23 ± 1.97 (mean ± SD) egg string aggregation per site, compared to only 0.40 ± 0.89 in 2017 and 0.28 ± 0.76 in 2018. In 2017, 73% of clutches (8 of 11) were infected across the 3 *Saprolegnia*-positive locations (range of *Saprolegnia* presence if egg clutches = 33–100%). In 2018, 2 sites showed signs of infections, with 1 infected egg clutch at 1 site (25% covered with *Saprolegnia*) and 3 egg clutches at the other site (average 40% covered with *Saprolegnia*). For the 2 yr combined, an average of 67.8% of the egg clutches (12 of 15) were infected at *Saprolegnia*-positive sites. The estimated percentage of successful hatching in infected egg clutches was 36% in 2017 and 25% in 2018, highlighting the fact that infection is not necessarily lethal.

Saprolegnia prevalence was significantly associated with the presence of adult, egg and tadpole *Bufo gargarizans* (Cochran's *Q* test; n = 17, $Q = 11.65$, df = 2, p = 0.003). For all years combined, there were adult *B. gargarizans* at 18.5% (n = 74) of sites without *Saprolegnia* versus 5.9% (n = 1) at sites with *Saprolegnia*. The difference was lower but opposite for eggs and tadpoles, although still marked by a roughly 2-fold difference, and similar between the 2 development stages. Across all 3 yr and 417 total surveys, eggs were present at 2.64% of sites (n = 11) with *Saprolegnia* versus 25.18% of sites without *Saprolegnia* (n = 105), while tadpoles were present at 2.64% of sites (n = 11) with *Saprolegnia* versus 22.78% of sites without *Saprolegnia* (n = 95).

3.2. Effect of *Saprolegnia* on breeding the subsequent year

Egg masses were found at 100% of sites (n_{sites} = 27) in 2016, 44.4% of sites (n_{sites} = 14) in 2017 and 55.6% of sites (n_{sites} = 15) in 2018. On average, *Saprolegnia* was found at 18.5% of sites (n_{sites} = 5) in 2016, 11.1% (n_{sites} = 3) in 2017, and 7.4% (n_{sites} = 2) in 2018, for an overall 11.1% at all 27 sites (Table 1). Egg presence differed between all years, but only differences between 2016 and 2017 were significantly explained by *Saprolegnia* presence, with fewer eggs during the latter year (repeated measures ANOVA, df = 1, χ^2 = 0.82, $F = 4.42$, p = 0.047). Breeding activity in 2017 and 2018 was not significantly explained by or correlated with *Saprolegnia* presence in the previous year (Table 2; Pearson's correlation, n = 27; 2017: r = 0.23, p = 0.239; 2018: r = 0.31, p = 0.108).

Table 1. Descriptive statistics of surveys conducted from 2016 to 2018 at 27 sites in Korea to investigate the impact of *Saprolegnia* on the breeding activity of *Bufo gargarizans*. *Considering detection of *Saprolegnia* at the same site several times during different surveys

	Total no. of surveys	Total no. of sites with eggs	*Saprolegnia*-positive sites	Records of *Saprolegnia* symptoms*	Egg string aggregations (mean ± SD)
2016	139	27	5	8	1.23 ± 1.97
2017	130	14	3	7	0.40 ± 0.89
2018	148	15	2	2	0.28 ± 0.76

Table 2. Results of the repeated measures ANOVA testing for variation in *Bufo gargarizans* egg presence at sites over years, and the relationship with the presence of *Saprolegnia* on egg strings. **Bold**: $p < 0.05$

	df	χ^2	*F*	p
Egg presence	1	2.02	16.34	**0.001**
Saprolegnia presence (2016)	1	0.63	3.38	0.079
Saprolegnia presence (2017)	1	0.82	4.42	**0.047**
Saprolegnia presence (2018)	1	0.10	0.56	0.460
Error	23	0.18		

3.3. Relationship between presence of *Saprolegnia* and ecological variables

Environmental variables and *Saprolegnia* prevalence were significantly correlated (n = 417; Omnibus test, $\chi^2 = 465.24$, df = 35, $p < 0.001$). Environmental variables collectively explained 93.0% of the variation in *Saprolegnia* presence (Nagelkerke R^2), but only the effects of water conductivity and habitat type were individually significant (binomial logistic regression; Table 3). *Saprolegnia* presence was positively associated with higher water conductivity (*Saprolegnia* prevalence: 368.33 ± 350.12 µS; absence of *Saprolegnia*: 183.48 ± 124.33; $p < 0.001$; Fig. 3). Regarding the type of habitats, *Saprolegnia* was present in 9.9% of ponds and 2.9% of lakes.

3.4. Molecular identification of *Saprolegnia*

The sequencing results identified the pathogenic oomycete as an undescribed *Saprolegnia* species in Korea within the *S. ferax* complex according to the Sandoval-Sierra et al. (2014) name system. Sequences from *B. gargarizans* eggs formed a clear monophyletic clade with *S. ferax* (99% support; Fig. 4). A BLAST search comparison with data from GenBank showed that the *Saprolegnia* we isolated was closely related to *S. ferax* from *Ambystoma gracile* (EU124763, 100%), *Anaxyrus boreas* (JQ974984, 99.8%), *B. calamita* (JX418014, 99.4%) and *Rana cascadae* (JQ974983, 100%) eggs. The isolates obtained fell within the 98.9 to 100% variation observed within the *S. ferax* cluster and were 96.9 to 97.2% similar to the closest species cluster, *S. delica.*

4. DISCUSSION

Our results showed that the prevalence of an undescribed strain of the pathogenic oomycete species *Saprolegnia ferax* was associated with an increased mortality in *Bufo gargarizans* eggs, as documented in other amphibians (Bragg & Bragg 1958, Bragg 1962, Blaustein et al. 1994, Kiesecker & Blaustein 1995, Perotti et al. 2013). While *Saprolegnia* presence in 2016 had a significant impact on the breeding activity of the subsequent year, no impact on the breeding activity of 2018 was found, and we cannot conclude on the impact of *Saprolegnia* on the subsequent breeding season of *B. gargarizans* at a site. On average, 67% of egg clutches were infected at sites where *Saprolegnia* was present in 2017 and 2018, similar to the 62.5% found in *Anaxyrus americanus* clutches in the USA (Gomez-Mestre et al. 2006). *Saprolegnia* was associated with a 43% mortality (range 25–75%) in

Table 3. Results of the binary logistic regression (*B*, n = 417) assessing for variables related to *Saprolegnia* presence on *Bufo gargarizans* egg strings in Korea between 2016 and 2018. Only water conductivity and habitat type are significant explanatory variables (**bold**, $p < 0.05$)

	B	SE	Wald	df	p
Year	0.03	0.19	0.021	1	0.884
Air humidity	−0.002	0.01	0.07	1	0.788
Water temperature	0.17	0.09	0.03	1	0.856
Water conductivity	0.05	0.03	3.53	1	**<0.001**
Water pH	0.20	0.39	0.25	1	0.615
Moon illumination	0.04	0.01	0.15	1	0.693
Photoperiod	0.01	0.01	1.82	1	0.177
Cloud cover	−0.01	0.01	0.18	1	0.667
Site(date)	−0.01	0.04	7.87	26	0.999
Habitat type	−9.54	394.86	0.01	1	**0.009**

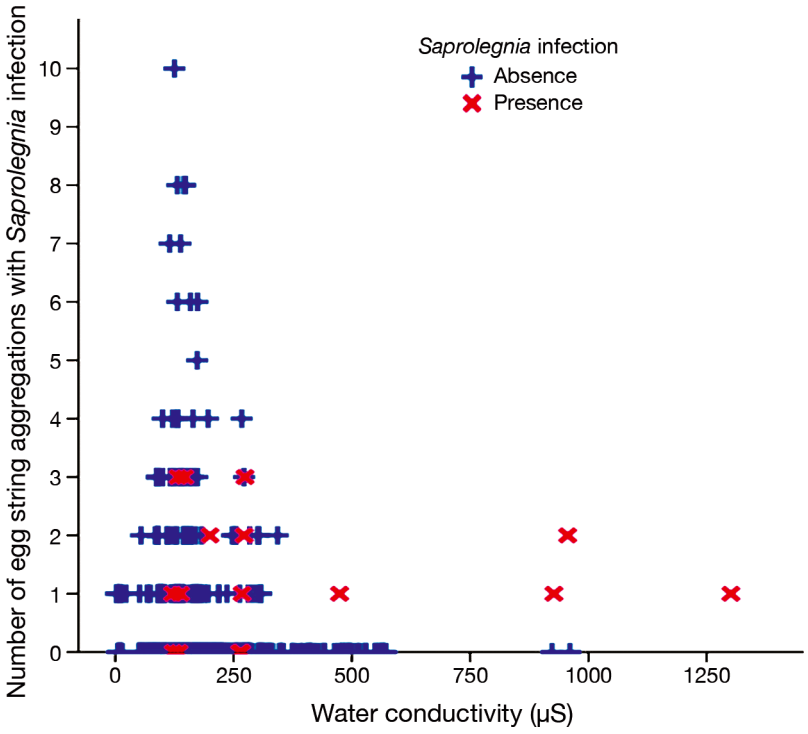

Fig. 3. Relation between water conductivity and *Saprolegnia* prevalence on *Bufo gargarizans* egg strings in Korea between 2016 and 2018. *Saprolegnia* presence was positively associated with higher water conductivity

infected clutches, which is also similar to the 25% average infection rate found for *A. americanus,* despite a larger reported range (range 5–90%; Gomez-Mestre et al. 2006). The increased mortality due to the infection may affect larvae and newly metamorphosed amphibians due to reduced size, as recorded in *Rana arvalis* (Uller et al. 2009). Uller et al. (2009) also suggest that besides embryonic mortality during *Saprolegnia* outbreaks, there could also be carry-over effects on several generations via offspring size and dispersal. Finally, the decrease in the number of sites where *B. gargarizans* was found breeding compared to the first year of surveys and the resulting inability to detect *Saprolegnia*, as there was no substrate for development, may have resulted in a variation in detectability. However, the higher prevalence detected in 2017 when there was a lower number of egg clutches compared to 2018 shows that the potential bias is negligible here.

B. gargarizans were not present at all survey sites in 2017 and 2018, even though they were in 2016. Similarly, it was hypothesized that breeding attempts of *B. periglenes* were discouraged because of unfavorable environmental factors in the water body (Crump et al. 1992). *Saprolegnia* infections are unlikely to have caused a nationwide decline of *B. gargarizans* populations since *Saprolegnia* prevalence was not related to breeding activities in 2017 and 2018 and was not detected in most of the study areas. Therefore, other factors such as site destruction, population decline, biennial breeding or unfavorable environmental factors at the breeding site, similar to *B. periglene*, might have played an important role at reducing breeding at these sites. Further research is needed to establish a causal relationship, though we suspect that population declines are most likely related to site destruction and unfavorable environmental factors. Some amphibian species do not breed every year in habitats with scarce resources (Morrison & Hero 2003), such as those in

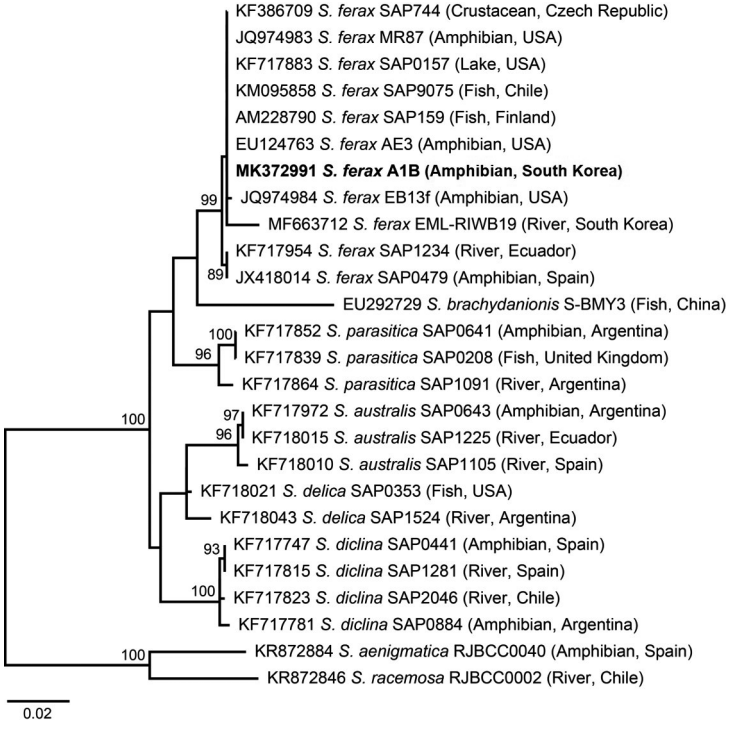

Fig. 4. Phylogenetic tree positioning *Saprolegnia* growing on egg strings of *Bufo gargarizans* (A1B) in Korea in the *Saprolegnia ferax* clade. The sequence of *Saprolegnia* was deposited in GenBank (MK372991)

northeastern Korea, where deforestation has negatively impacted populations (Park et al. 2014). However, meta-populations are made of several groups of individuals with different breeding dynamics; thus, even if one of these groups does not breed during one of the breeding seasons, it is still likely that breeding activity can be recorded at the site due to the presence of another group. This explains why breeding was recorded every year at sites within areas with large population sizes, such as those in the southern and western regions, while breeding was not recorded every year in the northeastern regions, where deforestation is a problem for amphibians (Park et al. 2014). In addition, climate change is most likely to impact even further the dynamic between *Saprolegnia* and egg development in *B. gargarizans* through the disruption of ecological variables (Daszak et al. 2003, Pounds et al. 2006, Bancroft et al. 2008, North et al. 2015) or through a distribution shift such as that seen in the sympatric species *Karsenia koreana* (Borzée et al. 2019a)

Environmental factors, such as water conductivity, play a role in *Saprolegnia* presence. High conductivity seems to be one of the preferred environmental factors for amphibians, e.g. *A. boreas* in the USA (Klaver et al. 2013), and plays an important role in osmoregulation and amphibian development (Cameron 1940, Morris & Tanner 1969, Hovingh 1993). However, in the present study, higher conductivity also seemed favorable for *S. ferax* (*Saprolegnia* presence: 368.33 ± 350.12 µS; *Saprolegnia* absence: 183.48 ± 124.33 µS). As expected, *B. gargarizans* was present in water with moderately high conductivity (median conductivity of this study = 148 µS), as observed with *A. boreas* in the USA. If a large breeding population, under higher conductivity conditions, was producing more eggs and resulting in a higher *Saprolegnia* infection rate, then *Saprolegnia* infection could be qualified as density dependent and not displaying direct preference for higher conductivity. However, increases in egg numbers at sites with higher conductivity were not significant in our study. Not all embryos develop equally, and within an egg clutch, some weaker embryos die from other causes (e.g. Tejedo 1992), without demonstrating density-dependent infection by *Saprolegnia*. It is suggested that toads in higher-conductivity water may cope better with stresses (Klaver et al. 2013); however, our results seem to show that higher than average conductivity has no beneficial effect for amphibian eggs against water mold infections like *Saprolegnia*. Our sites with high conductivity values (368.33 ± 350.12 µS) still had relatively moderate conductivity levels compared to the values presented in the literature (e.g. Karraker et al. 2008, Klaver et al. 2013, Borzée et al. 2018).

Ponds were significantly associated with *S. ferax* occurrence. *B. gargarizans* lay their eggs at the edge of ponds and lakes and often distribute egg strings around plants in the water. Ponds are shallower than lakes, which could affect the impact of UV-B radiation. While we did not detect any influence of photoperiod on *Saprolegnia* presence, high UV-B radiation is associated with a higher prevalence of *Saprolegnia* infections in amphibians (Kiesecker & Blaustein 1995), due to a synergistic interaction between the two. Separately, both UV-B radiations and *Saprolegnia* infections can damage amphibian embryos, and in an experimental setup, these factors had a synergistic damaging effect on amphibian embryos (Kiesecker & Blaustein 1995).

However, other factors could influence *Saprolegnia* infections in different habitat types. Larger water bodies act as buffers for daily variations in water quality, such as temperature and DO. Future studies should also consider the water depth where eggs are laid to test whether photoperiod or other variables have an impact on *Saprolegnia* prevalence and egg hatching success. Furthermore, the relatively thin gelatinous layer surrounding *B. gargarizans* egg strings may result in a weaker protection to *Saprolegnia* infection in comparison with other types of egg clutches (Blaustein et al. 1997, Green 1999, Gomez-Mestre et al. 2006). Additionally, infected eggs of *A. americanus*, similar to *B. gargarizans*, hatched earlier in the presence of *Saprolegnia*, and larvae were consequently exposed to higher predation risks (Gomez-Mestre et al. 2006).

Gomez-Mestre et al. (2006) showed that *Saprolegnia* grew 2.7 times faster in warmer water (11–15°C), where *A. americanus* breeds, than in colder water (6.5–8.5°C), where the Ranidae *R. sylvatica* breeds. Consequently, the difference in water temperature likely explains higher infection rates in *A. americanus* clutches (62.5%) than in *R. sylvatica* (7%; Gomez-Mestre et al. 2006). At our sample sites, *R. uenoi* and *Hynobius* spp. both started breeding earlier in the year and in colder water (Kim 2019) than *B. gargarizans*. The difference in water temperature during the breeding activities of these 2 groups of species likely results in a higher *Saprolegnia* infection risk for *B. gargarizans* egg clutches. Consequently, a slight increase in water temperatures in response to human-led environmental changes might lead to the geographic spread, increased occurrence and impact of *Saprolegnia*.

This scenario has already been proven right through ranavirus outbreaks (e.g. Bayley et al. 2013, Brand et al. 2016). Finally, the synergy with other arising threats to amphibians, such as predation (Bae et al. 2019) or invasive species (Brunner et al. 2015, Chuang et al. 2019, Groffen et al. 2019), may unbalance the relationship and result in an even faster decline of *B. gargarizans* populations.

The taxonomic status of several *Saprolegnia* species is controversial due to ambiguous morphological characteristics and a discrepancy between morphological and molecular information (Diéguez-Uribeondo et al. 2007, Sandoval-Sierra et al. 2014, Sandoval-Sierra & Diéguez-Uribeondo 2015). mOTUs based on the ITS region are useful tools to delimit *Saprolegnia* species (Sandoval-Sierra et al. 2014). Using these tools, we found a previously undescribed and not yet recorded strain of *S. ferax* (Fig. 4). *B. gargarizans* is the third amphibian species, besides *Pelophylax chosenicus* and *R. huanrenensis,* for which *Saprolegnia* infections have been recorded in Korea. In addition, it is important to highlight that only the *Saprolegnia* samples collected in 2017 were analyzed with molecular tools. While *Saprolegnia* sp. was confirmed through microscopic observation in 2016 and 2018, we cannot confirm that the strain present was the same as the one collected in 2017.

To conclude, our study shows that the water mold *S. ferax* is associated with high mortality rates in *B. gargarizans* egg clutches, and in combination with other factors, it could have a major impact on *B. gargarizans* populations, a species already declining because of urbanization-related habitat loss (Kuzmin et al. 2004). Furthermore, our results suggest a causal impact of high water conductivity and habitat type on *S. ferax* infection of *B. gargarizans* egg clutches. This deserves further study through manipulative experiments to determine causality.

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